REMARKS

Rejection under 35 USC §112, first paragraph

The specification as filed clearly supports the amended claims. According to the M.P.E.P. 2163:

> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., Vas-Cath, 935 F.2d at 1563, 19 USPQ2d at 1116; Martin v. Johnson, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in ipsis verbis [i.e., "in the same words"] to be sufficient").

The skilled worker would have recognized that the inventors had possession, at the time the application was filed, of a kit comprising a standard curve. Reagents kits commonly are sold with standard curves and/or reagents for creating standard curves. Even when a scientist is instructed to create his or her own standard curve, an example of a curve may be provided with the kit. For examples, see Exhibit A.

The use of "standard curves" is disclosed throughout the specification, e.g., Page 3, lines 17-20; Page 4, lines 5-8; Page 9, lines 4-7; Page 10, lines 10-15; Fig. 1. Furthermore, the standard curve is expressly referred to as being "previously determined" (Page 3, lines 17-20; Page 4, lines 5-8). Consequently, the skilled worker would have understood that such a standard curve could have been included in the kit, itself.

On Page 9 of the specification, an example is provided on how to measure a compound in accordance with the methods described in the specification. The example illustrates how a standard curve is prepared, and provides guidance on how an unknown is then subsequently measured. The direction on Page 10 ("just replace the standard sample by the sample to be determined, and read the unknown hirudin concentration in Fig. 1 from the measured reduction

of the optical density") informs the skilled worker that the unknown can be measured using the same procedure, but substituting it for the known. This example does not require that the standard curve always be performed in the same experiment.

The last sentence on page 10 of the present specification text may have been translated in a somewhat misleading way. A better translation would be: "In an experiment the standard sample is replaced by a sample to be determined and the unknown concentration of hirudin is read out in the Figure 1 with the measured reduction of the optical density". This sentence indicates that measuring an unknown sample is done in the same way as described for measuring the standard curve. The concentration in the unknown sample is then determined by entering the measured value of the reduction of the optical density into Figure 1, the standard curve.

Additional Claim 19 has also been added that recites that the kit comprises "a known amount of hirudin for determining a standard curve." Support for Claim 19 can be found throughout the specification, e.g., Page 8, lines 5-7; and Page 9, lines 4-7.

This application is a National Phase of WO0046602 which contained a Figure 1. This figure is attached for the examiner's convenience.

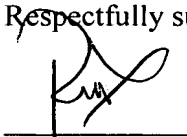
Rejection under 35 U.S.C. §102 and §103

Claims 9, 10, and 17 have been amended to recite the subject matter of claims 14, 16, and 18 which were not rejected under 35 U.S.C. §102 and §103. Therefore, it is believed that the amended claims are free of the cited prior art.

In view of the above remarks, favorable reconsideration is courteously requested. If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Date: August 25, 2004

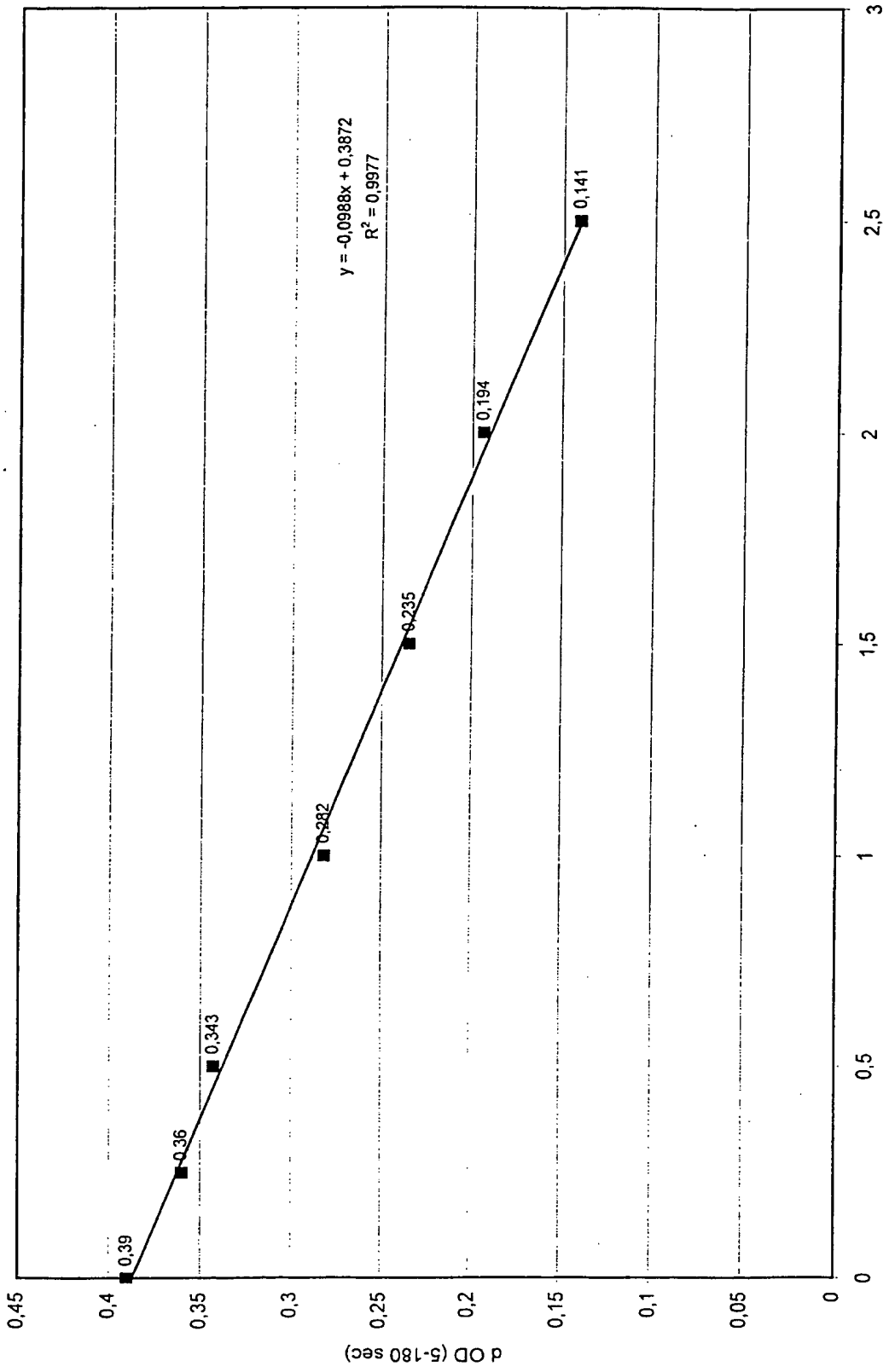

y = -0,0988x + 0,3872
R² = 0,9977
0,39
0,36
0,343
0,282
0,235
0,194
0,141
d OD (5-180 sec)
Hirudinkonzentration (µg/ml Plasma)
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0,5
1
1,5
2
2,5
3

Fig. 1

Performance Characteristics of the Avecon Diagnostics, Inc. Tetanus Antibody Test Kit

Sensitivity -

The sensitivity of the Avecon Tetanus Antibody Test was defined as the average plus three (3) standard deviations of a series of negative serum specimens (n = 20) run with the test kit. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

	Target Concentration (IU/mL)	Result (IU/mL)
	0	0
	0	1
	0	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	1
	0	0
	0	0
	0	0
	0	1
	0	1
	0	0
	0	0
	0	1
	0	0
	0	1
Average		0.35
Standard		0.48

Deviation		
3 S.D.		1.4

Based on these results, it was determined that the sensitivity of the Tetanus Antibody Test is 1.8 IU/mL tetanus antibody (0.35 + 1.4).

Linearity -

1. The linearity of the Avecon Tetanus Antibody Test was determined by running successively higher concentrations of tetanus antibody added to negative serum, and determining the concentration at which the curve ceased to form a straight line. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

Target Concentration (IU/mL)	Result (IU/mL)
10	10
15	15
25	25
50	56
75	90
100	111
125	111
150	107

Based on these results, it was determined that the Avecon Tetanus Antibody Test is linear up to 100 IU/mL tetanus antibody.

Precision -

The precision of the Tetanus Antibody Test was determined by:

1. Assaying the quantity of tetanus antibody in the tetanus antibody reference material provided by the National Institute of Biological Standards and Controls (NIBSC, United Kingdom), Lot # 76/589. Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.
2. Determination of inter- and intra- assay precision of the test using negative serum to which was added tetanus antibody at concentrations of 10, 15 and 25 IU/mL. Tetanus antibody concentrations were quantitated using the standard curve provided

with the kit.

Results

A. NIBSC Tetanus Antibody Standard Reference Material, Lot # 76/589 (n = 6) -

	Target Concentration (IU/mL)	Result (IU/mL)
	9.2	10
	9.2	10
	9.2	9
	9.2	9
	9.2	9
	9.2	8
Mean		9.2
Standard Deviation		0.69
C.V. (%)		7.5

B. Inter-Assay Precision - Tetanus antibody was added to negative serum at three (3) different concentrations (10, 15 and 25 IU/mL) and each were run on the same day (n = 20). Tetanus antibody concentrations were quantitated using the standard curve provided with the kit.

	10 IU/mL	15 IU/mL	25 IU/mL
	10	14	25
	10	14	25
	11	14	23
	10	15	25
	11	16	24
	10	14	23
	9	14	25
	11	15	24
	11	15	24
	10	14	25
	11	15	25

	10	15	26
	11	14	26
	10	15	24
	11	15	25
	11	15	25
	10	15	24
	11	14	24
	11	15	25
Mean	10.5	14.6	24.5
Standard Deviation	0.59	0.58	0.8
C.V. (%)	5.6	4	3.3

C. Intra-Assay Precision - Tetanus antibody was added to negative serum at three (3) different concentrations (10, 15 and 25 IU/mL) and each were assayed once per day for 15 days (n = 15). Tetanus antibody concentrations were quantitated using the standard curve provided with the kit. The same Tetanus Antibody Test kit was used for all analyses.

	10 IU/mL	**15 IU/mL**	**25 IU/mL**
	11	15	23
	11	15	25
	10	15	24
	10	15	24
	10	15	24
	10	14	25
	11	15	24
	11	15	24
	11	15	24
	11	16	24
	10	15	25
	10	15	25
	11	15	25
	11	15	24
	11	14	23
Mean	10.6	14.9	24.2

Standard Deviation	0.5	0.44	0.65
C.V. (%)	4.7	3	2.7

Interfering Substances -

The effects of interfering substances on the ability of the Tetanus Antibody Test to accurately determine the quantity of tetanus antibody in biological specimens was determined by the addition of triglycerides (750 mg/dL), hemoglobin (1000 mg/dL) and bilirubin (25 mg/dL) to a serum specimen containing 25 IU/mL tetanus antibody, and assaying the amount of tetanus antibody in the adulterated specimens using the standard curve provided with the kit. The results are as follows:

A. Triglycerides, 750 mg/dL (n = 7) -

	Target Concentration (IU/mL)	**Result (IU/mL)**
	25	22
	25	25
	25	28
	25	24
	25	27
	25	24
	25	26
Mean		25.1
Standard Deviation		1.9
C.V. (%)		7.6

B. Hemoglobin, 1000 mg/dL (n = 7) -

	Target Concentration (IU/mL)	**Result (IU/mL)**
	25	27
	25	28
	25	27
	25	28

	25	27
	25	27
	25	26
Mean		27.1
Standard Deviation		0.64
C.V. (%)		2.4

C. Bilirubin, 25 mg/dL (n = 7) -

	Target Concentration (IU/mL)	**Result (IU/mL)**
	25	24
	25	23
	25	24
	25	25
	25	24
	25	25
	25	25
Mean		24.3
Standard Deviation		0.7
C.V. (%)		2.9

Fluorokine® MAP Mouse JE/MCP-1 Kit

Catalog Number: LUM479
Pack Size: 100 Tests

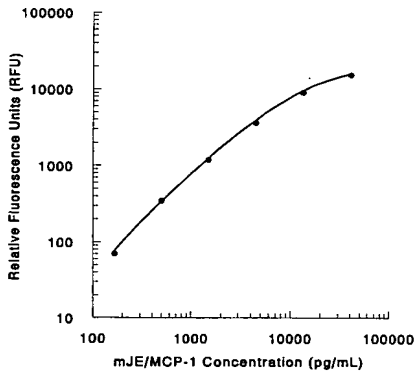
Specifications and Use

Recommended Sample Types	• Cell culture supernates, serum, and EDTA plasma
Microparticle Region	• Region - 30
Components	• Microparticle Concentrate (Part 892444) is supplied as a 100X concentrated stock (0.06 mL) with preservatives. • Biotin-Antibody Concentrate (Part 892459) is supplied as a 100X concentrated stock solution (0.06 mL) with preservatives.
Other Supplies Required	• Fluorokine MAP Mouse Base Kit (Catalog Number LUM000).
Storage	• Store unopened kit at 2 - 8° C. Do not use past the expiration date on the label. • **Avoid freezing microparticles.** • **Protect microparticles from light.**
Instructions for Use	• Refer to the Base Kit insert for the Fluorokine MAP assay procedure.

Typical Data

This mouse JE/MCP-1 standard curve is provided only for demonstration. A standard curve must be generated each time an assay is run, utilizing values from the Standard Value Card included in the Base Kit.

Note: *The Fluorokine MAP mouse JE/MCP-1 kit utilizes a six point standard curve. When fitting a standard curve constructed with the recommended 3-fold dilution series, use the first six points for the JE/MCP-1 kit only.*



Standard	pg/mL	RFU	Average	Corrected
Blank	0	21 21	21	—
1	40500	15302 15285	15293	15272
2	13500	9153 8936	9044	9023
3	4500	3674 3591	3632	3611
4	1500	1208 1222	1215	1194
5	500	434 301	367	346
6	167	89 92	90	69

Performance Characteristics

All data were collected with assays run as a multiplex.

Sensitivity - The Minimum Detectable Dose (MDD) was determined by adding two standard deviations to the mean RFU of twenty zero standard replicates and calculating the corresponding concentration.

Twenty-four assays were evaluated, and the MDD of mouse JE/MCP-1 ranged from 6.2 - 37.1 pg/mL. The mean MDD was 17.5 pg/mL.

R&D Systems, Inc.
1-800-343-7475

Intra-assay Precision (precision within an assay) - Two samples of known concentration were tested twenty times on one plate to assess precision within an assay.

Inter-assay Precision (precision between assays) - Two samples of known concentration were tested in twenty separate assays to assess precision between assays.

	Intra-assay Precision		Inter-assay Precision	
Sample	1	2	1	2
n	20	20	20	20
Mean (pg/mL)	894	3368	996	3592
Standard Deviation	24.1	99.9	91.2	233
% CV	2.7	3.0	9.1	6.5

Recovery and Linearity - Cell culture supernate, serum, and EDTA plasma samples were spiked with natural or recombinant mouse JE/MCP-1 and evaluated for recovery and were serially diluted to evaluate assay linearity.

Recovery			Linearity				
Sample	Average % Recovery	Range (%)			Cell culture supernates	Serum	EDTA Plasma
Cell culture supernates	95	77 - 110	1:2	Average % of Expected	112	100	94
				Range (%)	92 - 122	92 - 111	93 - 95
Serum	98	74 - 117	1:4	Average % of Expected	109	98	90
				Range (%)	92 - 116	90 - 133	87 - 92
EDTA Plasma	101	97 - 104	1:8	Average % of Expected	105	97	88
				Range (%)	89 - 117	86 - 121	86 - 89

Specificity - This assay recognizes both natural and recombinant mouse JE/MCP-1. The assay was tested for interference and cross-reactivity with over 80 related factors. Less than 0.5% cross-reactivity and interference was observed.

Recombinant mouse:		**Recombinant human:**		**Recombinant rat:**	**Recombinant mouse Multiplex partners:**
C10	LIF	GM-CSF	IL-10	CNTF	GM-CSF
CTACK	MARC/MCP-3	IFN-γ	IL-12 p35	GM-CSF	IFN-γ
Eotaxin	MCP-5	IL-1β	IL-12 p40	IFN-γ	IL-1β
FAS Ligand	M-CSF	IL-1 Rα	IL-12 p70	IL-1α	IL-2
G-CSF	MDC	IL-1 sRI	MCP-1	IL-1β	IL-4
IL-1α	MIP-1α	IL-1 sRII	MCP-2	IL-2	IL-5
IL-1 Rα	MIP-1β	IL-2	MCP-3	IL-4	IL-6
IL-3	OPN	IL-2 sRα	TNF-α	IL-5	Il-10
IL-7	OPG	IL-2 sRβ	TNF sRI	IL-6	IL-12 p70
IL-9	OSM	IL-4	TNF sRII	IL-10	IL-13
IL-10 sRα	P*l*GF-2	IL-4 sR	VEGF	Leptin	KC
IL-12 p40	RANTES	IL-5		TNF-α	MIP-2
IL-17	SCF	IL-5 sR		$VEGF_{164}$	TNF-α
IL-18	TECK	IL-6			VEGF
Leptin	TNF sRII	IL-6 sR			
	TPO				
	TRANCE				

Technical Hints:

- Protect the microparticles and streptavidin-PE from light at all times.
- Refer to the Base Kit Standard Value Card for reconstitution volume and value of the reconstituted standard.
- Diluted microparticles cannot be stored. Make a fresh dilution of microparticles each time the assay is run.
- The use of a vacuum manifold device made to accommodate a microplate is necessary for washing. Adjust the vacuum to between 15 and 40 cm Hg.
- Discrepancies may exist in values obtained for the same analyte utilizing different technologies.

Fluorokine MAP affords the user the benefit of multianalyte analysis of cytokines in a complex sample. For each sample type, a single, multipurpose diluent is used to optimize recovery, linearity and reproducibility. Such a multipurpose, single diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.

COATEST®
LMW Heparin / Heparin

Art. No. 82 13 63

CHROMOGENIX

Chromogenix-
Instrumentation Laboratory SpA
V. le Monza 338 - 20128 Milano (Italy)

302103R0

ENGLISH

COATEST®
LMW Heparin / Heparin

For In Vitro Diagnostic Use

INTENDED USE OF THE KIT
For the in vitro photometric determination of the heparin or LMW heparin activity in human plasma.

MEASUREMENT PRINCIPLE

1. LMW Heparin + AT ⟶ [LMW Heparin · AT]

2 a. [LMW Heparin · AT] + FXa (excess) ⟶ [LMW Heparin · AT· FXa]

2 b. S-2732 + FXa ⟶ Peptide + pNA (yellow) + FXa (residual)

LMW Heparin (e.g. Fragmin) or Heparin is analyzed as a complex with Antithrombin (AT) present in the plasma sample. Activated Factor X (FXa) is added to a mixture of plasma sample and the chromogenic peptide substrate S-2732 in a buffer. Two competing reactions then start. One is the inhibition of FXa by the [LMW Heparin · AT] complex, the other is the FXa catalyzed release of pNA from the substrate. After a certain period of time most of the FXa is inhibited and the release of pNA has essentially declined. Further release of pNA is stopped by the addition of acetic acid and the absorbance at 405 nm is measured. The correlation between absorbance (A 405) and LMW Heparin/Heparin activity is linear in the 0.1–1.0 IU/ml range when plotted in a log-lin scale.

REAGENTS
The sealed reagents are stable at 2-8°C until the expiration date printed on the label. Avoid contamination by microorganisms of the reagent.

1. **S-2732** 1 vial
   Chromogenic substrate (Suc-Ile-Glu(γ-Pip)-Gly-Arg·pNA), 6 mg, with mannitol as bulking agent. Reconstitute with 2.6 ml water to a concentration of 2.9 mmol/l. The solution is stable for 6 months at 2-8°C.
2. **Factor Xa** 1 vial
   Bovine Factor Xa, 13 nkat. Reconstitute with 10.4 ml water. The solution is stable for one month at 2-8°C.
3. **Buffer, 20 ml** 1 vial
   Tris 50 mmol/l, EDTA 7.5 mmol/l, pH 8.4, I=0.2. Once opened, the buffer solution is stable for two months at 2-8°C.
4. **LMW Heparin Standard, 1ml** 1 vial
   A LMW Heparin (Fragmin) standard, 100 IU/ml. (Calibrated against the 1:st International Standard for LMW Heparin, established by the WHO, using an anti-Factor Xa method). Once opened, the solution is stable for 6 months at 2-8°C.

Reagents required but not provided
- Deionized water, filtered through 0.22 µm or NCCLS type II water.[5]
- Saline (0.9% NaCl).
- Pooled normal human plasma taken on ice and prepared according to "SPECIMEN COLLECTION." A lyophilized preparation is available from Chromogenix AB or subsidiaries.
- Stopper solution: acetic acid 20% or monosodium citrate 20%.

Materials required but not provided
- Photometer, 405 nm
- Semi-micro cuvette (1cm)
- Centrifuge, 2000-4000 x g
- Stopwatch
- Disposable plastic tubes
- Pipettes, calibrated

SPECIMEN COLLECTION
Nine parts of freshly drawn venous blood are collected into one part trisodium citrate. Centrifugation: 2000 x g for 10-20 minutes at 20-25°C. Refer to NCCLS document H21-A2 for further instructions on specimen collection, handling and storage.[5]

PROCEDURE – MANUAL TECHNIQUE
Precautions
Proper mixing is important to make sure that the reaction mixture is homogenous, but avoid vigorous mixing as the proteins may precipitate in the foam. The method is designed for room temperature and should be kept within ±2°C from the temperature used when the standard curve was established.

CALIBRATION
A standard curve is required for each new lot of the Coatest LMW Heparin/Heparin.

LMW Heparin
Add 10 µl of the LMW Heparin standard (100 IU/ml) to 1.0 ml of pooled normal plasma and mix carefully to obtain a plasma containing 1.0 IU/ml. Prepare standards by mixing this 1.0 IU/ml plasma solution with pooled normal plasma according to the table below.

Heparin
For the determination of heparin, the standard curve must be made up by using a heparin standard of known concentration (not provided). Dilute the heparin with saline in order to obtain 100 IU/ml and proceed according to the instructions for LMW Heparin above.

Plasma Standards IU/ml	Spiked plasma 1.0 IU/ml µl	Pooled normal plasma µl
0.1	25	225
0.3	75	175
0.5	125	125
0.8	200	50
1.0	250	0

These standards can be kept at –20°C or below for 6 months or at 2-8°C for two days. For routine purposes larger amounts can be produced and kept frozen in suitable aliquots.

Quality control
It is suggested that each time a test is performed one should include a LMW Heparin/Heparin plasma sample preparation, other than that used to create the standard curve. This could be a single sample obtained from any source and at a known concentration about midrange within the standard curve.

Method
Perform at room temperature (20–25°C).
S-2732 + Buffer solution
Mix 1 volume of substrate with 3 volumes of buffer. The solution is stable for at least 24 hours at room temperature or one week at 2-8°C.

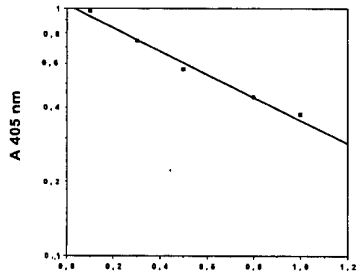
Add in a test tube	Sample or standard	Plasma blank (Note 1)
S-2732 + buffer	200 µl	–
Buffer	–	400 µl
Standard or test plasma	25 µl	25 µl
Factor Xa	200 µl	–
Mix and incubate for 8 min	–	
Stopper solution (Note 2)	200 µl	200 µl

Mix.
Read the absorbances at 405 nm. The colour is stable for at least 4 hours.
Substract the absorbance of the plasma blank from the sample.

Note 1. If the sample is not significantly more opaque than the standard plasma and if the bilirubin or haemoglobin content are not high enough to interfere, the pooled normal plasma blank can be used for all samples.

Note 2. The volume of the stopper solution can be chosen between 200–1000 µl in order to fit the cuvette.

CALCULATION
Plot the absorbance (A 405) for the standards (Y axis) after subtracting the plasma blank against their respective concentration of LMW Heparin/ Heparin (X-axis) on log/lin graph paper. Check whether A 405 for the two control samples correspond with the standard curve for the lot number of the kit. (Each laboratory must set it´s own guideline for control of assay quality). Read the IU/ml value for the unknown plasma from the standard curve after substracting the A 405 for the plasma blank.



LIMITATIONS
1. Valid determination of activities below 0.05 IU/ml may be difficult due to influence from heparin antagonists released from the platelets. Such low levels are, however, generally considered to be of limited clinical relevance.
2. If the sample contains more than 1.0 IU/ml, dilute 1:3 in pooled normal plasma and repeat the assay. Multiply the result by 3.

Expected results
To obtain an optimal effect with minimum risk of bleeding the LMW Heparin/Heparin activity should be in the range recommended by the producer. Suitable time point for sampling (also stated by the producer) must be considered.

Precision
The coefficient of variation (C.V.) within serie (n=15) was 0.8% (0.8 IU/ml) and 3.3% (0.4 IU/ml). The following table shows the C.V. between series at various activities. 15 assays were performed during 4 consecutive weeks with reconstituted reagents kept at 2-8°C.

IU/ml	0.2	0.4	0.6	0.8	1.0
Heparin C.V.	6.9%	4.2%	5.7%	6.9%	8.0%
Fragmin C.V.	6.5%	5.9%	3.9%	5.7%	7.2%

ACCURACY
The assay correlates well with Coatest Heparin. Plasma samples drawn from healthy volunteers after intravenous injection of Fragmin gave a correlation coefficient of 0.98 in the activity range 0–3.5 IU/ml (n=439). In patients treated with s. c. Fragmin activities in the range 0–1.2 IU/ml gave a correlation coefficient of 0.98 (n=56).

Sensitivity
The assay allows detection of 0.05 IU/ml of LMW Heparin/Heparin. To increase the accuracy in the range below 0.1 IU/ml a 0.05 IU/ml standard is recommended.

Specificity
The assay measures specifically the anti-Factor Xa effect of LMW Heparin/Heparin. The method is slightly dependent on the patients antithrombin concentration, since antithrombin is essential for the effect of LMW Heparin/Heparin. If the result obtained deviates from the expected activity, measurement of patients antithrombin level is recommended (Coatest Antithrombin or Coacute Antithrombin available from Chromogenix or subsidiaries).

REFERENCES

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3. HA Holm et al. Heparin assays and bleeding complications in deep venous throm-bosis with particular reference to retroperitonal bleeding. Thromb Haemostas 53, 278–281(1985).
4. E Holmer et al. A new simple chromogenic assay for Heparin and Heparin-like anti- FXa activity in plasma. Thromb Haemostas 54, 29 (1985).
5. AN Teien & M Lie. Evaluation of an amidolytic Heparin assay method: Increased sen-sitivity by adding purified antithrombin III. Thromb Res 10, 399–410 (1977).
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8. National Committee for Clinical Laboratory Standards. Specifications for reagent water used in the clinical laboratory, NCCLS Approved Standard: ASC-3.
9. National Committee for Clinical Laboratory Standards.
   Collection, transport and processing of blood specimens for coagulation testing and performance of coagulation assays,
   NCCLS Document H21-A2; vol. 11 No. 23

CHROMOGENIX

302103R0

Enzyme Immunoassay for the Quantitative Determination of Thyroxine (T4) in Human Serum

Catalogue #KH7010

Contents

Intended Use

For the quantitative determination of thyroxine (T4) in human serum. This kit is not licensed for use as a diagnostic test in the United States. It is therefore only sold outside the United States and is marked "for export only."

Outside the United States, the appropriateness of this test kit for research or diagnostic purposes depends on local regulations.

Return to top of page **Return to EIA list** **Return to Home Page**

Introduction

L-Thyroxine (T4) is a hormone that is synthesized and stored in the thyroid gland. Proteolytic cleavage of follicular thyroglobulin releases T4 into the bloodstream. Greater than 99% of T4 is reversibly bound to three plasma proteins in blood - thyroxine binding globulin (TBG) binds 70%, thyroxine binding pre-albumin (TBPA) binds 20%, and albumin binds 10%. Approximately 0.03% of T4 is in the free, unbound state in blood at any one time.

Diseases affecting thyroid function may present a wide array of confusing symptoms. Measurement of total T4 by immunoassay is the most reliable and convenient screening test available to determine the

presence of thyroid disorders in patients. Increased levels of T4 have been found in hyper-thyroidism due to Grave's disease and Plummer's disease and in acute and subacute thyroiditis. Low levels of T4 have been associated with congenital hypothyroidism, myxedema, chronic thyroiditis (Hashimoto's disease), and with some genetic abnormalities.

Return to top of page **Return to EIA list** **Return to Home Page**

Principle of the test

In the T-4 EIA, a certain amount of anti-T-4 antibody is coated on microtiter wells. A measured amount of patient serum, and a constant amount of T-4 conjugated with horseradish peroxidase are added to the microtiter wells. During incubation, T-4 and conjugated T-4 compete for the limited binding sites on the anti-T-4 antibody. After a 60 minutes incubation at room temperature, the wells are washed 5 times by water to remove unbound T-4 conjugate. A solution of TMB is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped and the color is changed to yellow with the addition of 2N HCl. The extent of color development is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled analyte in the sample.

Return to top of page **Return to EIA list** **Return to Home Page**

Materials

Materials provided with the test kits:

1. Antibody coated microtiter plate with 96 wells.
2. Reference standard set ready to use: 0, 1, 2.5, 5, 15, and 30.
3. Enzyme Conjugate Reagent.
4. Color Reagent A.
5. Color Reagent B.
6. 2N HCl.

Materials required but not provided:

- Precision pipettes: 50µl, 100µl, 200µl, 1ml, and 5ml.
- Disposable pipette tips.
- Distilled water.
- Glass tubes or flasks to mix Color Reagent A and Color Reagent B.
- Absorbent paper or paper towel.
- Graph paper.

Return to top of page **Return to EIA list** **Return to Home Page**

Instrumentation

The following equipment items are required to perform this assay:

- A vortex mixer or equivalent to mix reagents.
- A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-2 OD or greater at 450nm wavelength is required for use in the absorbance measurement.

Return to top of page Return to EIA list Return to Home Page

Storage of test kits

Unopened test kits should be stored at 2-8°C upon receipt. The microtiter plate should be stored at 2-8°C in a sealed bag with desiccants. This will minimize its exposure to damp air. Opened test kits will remain stable until the expiration date, provided they are stored as described above.

Return to top of page Return to EIA list Return to Home Page

Specimen collection and preparation

1. This kit is for use with serum samples prepared from whole blood. Blood should be drawn using standard venipuncture techniques and the serum should be separated from the red blood cells as soon as practical. Avoid grossly hemolytic, lipemic or turbid samples.
2. Plasma samples collected in tubes containing EDTA, heparin, or oxalate may interfere with the test procedures and should be avoided.
3. Specimens should be capped and may be stored up to 48 hours at 2-8°C, prior to assaying. Specimens held for a longer time can be frozen at -20°C. Thawed samples must be mixed prior to testing.

Return to top of page Return to EIA list Return to Home Page

Reagent preparation

1. All reagents should be brought to room temperature (18-25°C) and mixed by gently inverting or swirling prior to use. Do not induce foaming.
2. Add 1 ml of distilled water to reconstitute the lyophilized standards. Allow the reconstituted materials to stand for at least 20 minutes. Mix gently . The reconstituted standards should be stored sealed at 2-8 °C.
3. To prepare TMB substrate reagent, , make a 1:1 dilution of Color Reagent A and Color Reagent B at least 15 minutes before use. Mix gently to ensure complete mixing. The prepared TMB substrate reagent is stable at room temperature, in the dark, for up to 3 hours. Discard excess after use.

Return to top of page Return to EIA list Return to Home Page

Assay procedures

1. Secure the desired number of coated wells in the holder.
2. Dispense 50µl of standard, specimens, and controls into appropriate wells.
3. Dispense 100µl of Enzyme Conjugate Reagent into each well.
4. Thoroughly mix for 10 seconds. It is very important to have complete mixing in this step.
5. Incubate at room temperature (18-25°C) for 60 minutes. ***Prepare TMB solution 15 minutes before use.***
6. Remove the incubation mixture by flicking plate content into a waste container.
7. Rinse and flick the microtiter wells 5 times with running tap or distilled water.
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 200µl of TMB solution into each well. Gently mix for 5 seconds.
10. Incubate at room temperature for 20 minutes without shacking.
11. Stop the reaction by adding 50µl of 2N HCl to each well.
12. Gently mix for 30 seconds to make sure that the blue color changes to yellow color completely.
13. Within 30 minutes, read the optical density at 450nm with a microtiter plate reader.

Important Notes

- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
- It is recommended that no more than 32 wells be used for each assay run if manual pipetting is used since pipetting of all standards, specimens and controls should be competed within 3 minutes. A full plate of 96 well may be used if automated pipetting is available.
- Duplication of all standards and specimens, although not required is recommended.

Return to top of page **Return to EIA list** **Return to Home Page**

Calculation of results

Calculate the mean absorbance value (A_{450}) for each set of reference standards, specimens, controls and patient samples. Construct a standard curve by plotting the mean absorbance obtained from each reference standard (Y-axis) against its concentration (X-axis) on graph paper. Use the mean absorbance values for each specimen to determine the corresponding concentration of T4 from the standard curve.

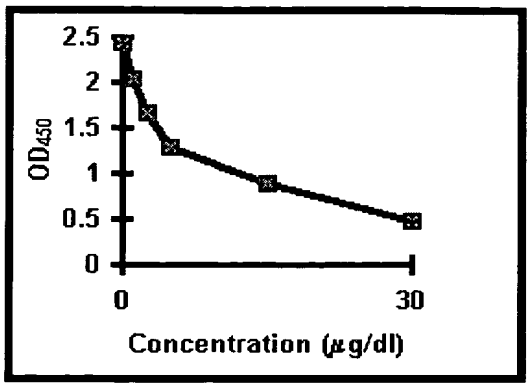
Return to top of page **Return to EIA list** **Return to Home Page**

Example of Standard Curve

Results of a typical standard run with the optical density reading at 450nm shown in the Y-axis against the T4 concentrations shown in the X-axis.

Note: *This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.*

T4 (µg/dl)	Absorbance (450nm)
0	2.44
1	2.05
2.5	1.66
5	1.29
15	0.89
30	0.48



Return to top of page Return to EIA list Return to Home Page

Expected values and sensitivity

T4 EIA was performed in a study of 200 euthyroid patients in one geographic location and yielded a normal of 5.0 to 13.0 µg/dl. This range corresponds to those suggested by other commercial manufacturers. It is recommended that laboratories adjust values to reflect geographic and population differences specific to the patients they serve. The minimum detectable concentration of thyroxine by this assay is estimated to be 0.4µg/dl.

Return to top of page Return to EIA list Return to Home Page

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Return to top of page Return to EIA list Return to Home Page

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